

PROBLEMS ASSOCIATED WITH THE DETERMINATION OF MONOCLONAL IMMUNOGLOBULINS AND THEIR FRAGMENTS IN PLASMA CELL DYSCRASIAS*

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Abstract

In 1978, a long term study of plasma cell dyscrasias began at the University Hospital, Kuala Lumpur. This article reports the problems (and their solution) associated with immunological estimations in such patients. The methods used included agar and agarose electrophoresis and immunoelectrophoresis and immunofixation as well as other methods in serum and urine. Results indicate that agarose gel electrophoresis instead of cellulose acetate electrophoresis should be used for the screening of sera for monoclonal immunoglobulins and/or their fragments. The resolution potential of immunoelectrophoresis appears to be dependent on the materials used, however, immunofixation was more sensitive in some cases.

INTRODUCTION

The determination and typing of monoclonal immunoglobulins is of diagnostic and prognostic importance: IgG myelomas are generally associated with a greater reduction of other classes of polyclonal immunoglobulins, more episodes of infection, slower tumour growth rate, decreased incidence of hypercalcaemia, amyloidosis and hyperviscosity syndrome whereas hypercalcaemia and amyloidosis are more common in patients with IgA myeloma. Patients with light chain disease (Bence Jones myeloma) usually present earlier, have a faster tumour growth rate, more osteolytic lesions, increased incidence of hypercalcaemia, renal failure, and amyloidosis and have poorer prognosis than IgG or IgA myeloma patients. IgD

myeloma patients also present earlier and have a poorer prognosis than IgA or IgG myelomatosis.^{1,2} Patients with lambda type light chain disease had a much lower median survival time in the Medical Research Council Myeloma Trial than kappa type.³

Paraproteins, M-proteins, M-components or more correctly monoclonal immunoglobulins (MIg) may appear as discrete bands on cellulose acetate zone electrophoresis (CAE). However, agarose gel electrophoresis (AGE) is at present the zone electrophoresis method of choice.⁴ Immunofixation (IMF)⁵ appears to be better for the classification and typing of MIg than classical immunoelectrophoresis (IEP).⁶

IEP is the standard technique for characterising MIg, however, its resolution power is relatively poor. Hence certain MIg and their fragments are difficult both to detect and to type.⁸ Another disadvantage of IEP is the four to five days required for its completion including staining.

The determination of Bence Jones proteins is also beset with problems particularly their sensitivity to bacterial contamination and prozoning.⁹

Other problems associated with the determination of MIg include lack of standardization and variance in quality of agar, agarose and commercially available antisera and antigenic specificity of such antisera, and collection of samples.

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This paper will discuss our approaches to the above problems.

MATERIALS

Antisera

The following antibodies were purchased from Behringwerke AG, Marburg, W. Germany: a trivalent antisera (anti-GAM) specific for heavy and light chains (from goat (g) OTEB 05); monovalent antisera specific for λ chains (from rabbit (r) ORCM 05, g OSBB 05), α chains (r RCI 05, porcine (p) OSBA 05), μ chains (r RCK 05, g OTPT 05), δ chains (r OTND 05), ϵ chains (r OTNP 05), κ chains bound (r OTNL 05), κ chains free (g OTNN 05), h chains bound (r OTNM 05), h chains free (g OTNO 05); from Meloy Laboratories Inc., Springfield, U.S.A.: anti-bound and free κ chains (A111), antibody and free h chains (A118); from Cappel Laboratories, Cochranville, U.S.A.: anti-bound and free κ chains (no code given) and from Helena Laboratories, Beaumont, U.S.A. γ chains (9232), μ chains (9234), ϵ chains (9250), κ chains (9231).

Controls

For heavy chains: Standard Human Serum, stabilized (ORDT 03), Behringwerke.
Pooled serum from at least 10 healthy blood donors.

For light chains: Bence Jones kappa and lambda Controls (OTEU 03 and OTEV 03) Behring.

For methods: IEP Control Set (A 200). Meloy.

Rid-plates

Tripartigen IgG, IgA and IgM (OTDS 03, OTDT 03, OTDU 03), Behring.

Agars

Purified Agar (L 28, lot no. 265 8272) Oxoid Ltd., London, U.K. Agarose (A-6877, lot no. 24C0550) Sigma Chemical Co., St. Louis, U.S.A. Agarose Type L (OREO 15, lot no. 252006B) and Type T (ORGT 15, lot no. 2601A), Behring.

Equipment

Sepratek equipment for CAE, Gelman Instrument Co., Ann Arbor, U.S.A. Shandon equipment for IEP, Vokam Power Pack, London, U.K. Universal electrophoresis equipment and cooling bridge and rectifier from Behring.

Buffers, etc

1. Barbitol buffer pH 8.6, ionic strength 0.02: 41.2 g sodium barbitone, 8.0 g barbitone, up to 10 liters with distilled water.
2. Diethylbarbiturate-acetate buffer, pH 8.6, ionic strength 0.1: 13.38 g sodium barbitone, 8.83 g sodium acetate. $3H_2O$, up to 1.5 liters with distilled water; 0.1 N hydrochloric acid (about 180 ml) until pH 8.2.
3. TRIS-barbitol buffer pH 8.6, ionic strength 0.02: 44.3 g TRIS, 22.4g barbitone, 0.53 g calcium lactate, 1.0 g sodium azide (optional), deionized water to 1 liter; dilute 1 + 4 before use.
4. Picric-acetic acid fixer: 14 g picric acid + 1 liter distilled water giving a saturated solution; after warming solution to 35 – 40°C, filter and add 200ml glacial acetic acid.
5. Coomassie stainer: 5 g Coomassie Brilliant Blue R, 450 ml ethanol 95%. 100 ml glacial acetic acid, 450 ml deionized water. Mix all except water, leave at room temperature for one day, filter and then add the water.
6. Differentiating destainer for 5 : As for 5 without dye.
7. Amido black stainer: 5 g amido black, 700 ml ethanol 95%, 200ml deionized water, 100 ml glacial acetic acid, mix overnight and filter before use.
8. Differentiating destainer for 7 : As for 7 without dye.

Sample collection

Serum was collected wherever possible in sterile containers under aseptic conditions to prevent bacterial contamination. Twenty-four hour urines were collected in sterile bottles containing 1 g/liter of sodium azide.

Urine concentration

Urines were tested at various concentrations: 1:1 (neat), 1:30, 1:100 and sometimes higher. Urines were concentrated using Ultra-thimbles, type 10, from Schleicher and Schuell, W. Germany, using the appropriate apparatus from the same manufacturer.

METHODS

Cellulose-acetate Microzone Electrophoresis: was carried out according to the manufacturer's instructions using Sepratek equipment (Gelman Instrument Company, Ann Arbor, USA).

Immuno-electrophoresis: was performed according to Table 1 which compares our original method to the method we are currently using.

Agarose Gel Zone Electrophoresis (Fig 1): was performed as described by Pedersen and Axelsen¹⁰ with modifications: 13mls of 1% w/v agarose gel in TRIS-barbital buffer was applied to 100 x 100 mm glass plates which had been stored under acetone. 5 µl sera or standard previously diluted 1:5 or higher with buffer was pipetted into slits cut into the gel, electrophoresis was carried out at 16 V/cm until a bromophenol-stained albumin marker had migrated 5 cm (usually 280 V, 30 mA for 50-55 minutes). The gels were then fixed for 10 minutes in picric-acetic acid fixer, washed in 95% alcohol for 5 minutes, dried and stained with Coomassie stainer, differentiated and dried. To obtain better contact between the buffer and the agarose gel, a simple method of making agarose gel bridges was developed as follows: Whatman's No. 1 filter paper, cut to 100 x 100 mm, was moistened in buffer and laid flat avoiding air bubbles on a 100 x 100 mm fat-free glass plate; 15 ml of a 1% agarose (Sigma) solution was poured onto the latter on a horizontal levelling table. A further piece of filter paper was added to the surface of the gel after cooling.

Immuno-fixation (Fig 2): was carried out as described by Pedersen and Axelsen¹⁰ with modifications: Sepraphore III cellulose acetate strips (Gelman) cut 57 x 10 mm were soaked in undiluted antibody and laid cathodally on the relevant section of a glass

plate covered with agarose gel that had been subjected to agarose gel electrophoresis as described above but not fixed, and incubated for one hour in a humidity chamber. The strips were then removed and the gels were squeezed under filter paper⁴ for 15 minutes, washed for 15 minutes in 2% w/v saline and further compressed for 15 minutes; this was then repeated twice with deionized water, dried and stained with Coomassie stainer.

RESULTS AND DISCUSSION

Occasionally a situation exists where the radiological, clinical and biopsy findings are strongly indicative of myeloma, Waldenstrom's macroglobulinaemia or some other plasma cell dyscrasia (PCD) but the immunochemical findings in serum or urine are negative. This seems to be due to a lack of sensitivity of our methods, to prozoning or poor sampling but probably not to a higher incidence of non-secreting PCD in our hospital population.

In a series of 69 PCD patients from previous years,¹ it was found that 18 patients did not show a discrete M-band or spike on CAE and would have been missed had the clinical and radiological evidence not been so strong. All of these patients were shown to have MIg or their fragments in serum by IEP. The questions then arose: were we missing identifying MIg in other patients where the clinical evidence for PCD was weak or inconclusive because the CAE was negative?

Recently, the sera of 15 patients with proven PCD (myeloma, macroglobulinaemia and lymphoma) were found to have discrete bands in 13 sera by AGE but only in 10 by CAE. MIg were identified in all sera by IMF and in 11 sera by IEP. The two sera identified by IMF but not by AGE were from patients with kappa and lambda light chain disease respectively.

The change of IEP method (Table 1) has increased the definition of the precipitin arcs and reduced flaring at their ends in normal sera as well as providing a better standardisation of the procedure. The use of agar for heavy chain typing and agarose for light chain typing as well as determining the presence of free light chains has assisted in picking up trace, abnormal accessory and split precipitin lines. Nevertheless,

Table 1 Comparison of old and current methods used for immunoelectrophoresis.

METHOD:	OLD	CURRENT
gel	agarose (Sigma) 2%	for H-chains agar (Oxoid) 1.5% for L-chains agarose L (Behring) 1.5%
gel volume	26 ml per Shandon slide carrier	2.5ml per glass slide 1" x 3"
sample volume	fill well	0.002 ml
antisera volume	fill trough	0.05 ml
buffer	barbiturate-lactate pH 8.6	barbiturate-acetate pH 8.2, fresh. μ 0.1 for tanks, μ 0.05 for gel
run	300 V constant, Vokam rectifier	6 V/cm with cooling, constant voltage Behring rectifier
time	90 minutes	45 minutes
wicks	Whatman's No.1 filter paper	Whatman's chromatography paper No. 3 MM
stain	amido black 0.1%	Amido black 0.5% or Coomassie stainer

the latter general rule cannot be applied to 'problem' sera. Figs 3 - 7 show the results obtained when the gel media and antisera were varied but otherwise using the same conditions as listed in Table 1; slide coding: left hand side - AO = Oxoid purified agar, SA = Sigma agarose, LA Behring agarose type L ; right hand side - patient's number or initials, H = Helena or B = Behring antisera as follows: 1 = IgG, 2 = IgA, 3 = IgM (where three precipitates trivalent against IgG + IgA + IgM), 4 = kappa bound, 5 lambda bound, 6 kappa free. The abnormal IgG kappa precipitin line is best defined in slides LASWB1 (gamma chain) and SASWB4 (kappa chain) in Fig. 4 whereas the IgG kappa line from another patient is best shown in slides LA1682B1 and LA1682B4 (Fig. 5). The abnormal kappa line in Fig. 6 can only be identified in slide OA1375B4 and the mu chain MIg is best seen in slide LA1683H3 (Fig. 7).

Considering the heterogeneity, the classes and

subclasses, the light chain types and subtypes as well as phenotypes and idiotypes of immunoglobulins it is not surprising that different commercial antisera react differently with different MIg. A battery of antisera should thus always be used. Meloy kappa and lambda light chain monospecific antisera, Behringwerke free light chain antisera, anti-IgG from rabbit, anti-IgA from pig and IgM from goat gave the best results when tested against the IEP sera supplied by Meloy and patient sera but no antisera was universal in its reaction or application. Standardized monospecific antisera produced against a large pool of MIg as well as divalent antisera against kappa and lambda bound light chains and another against the free forms would be welcome for IMF.

Staining and washing time was reduced by using the compression technique described permitting completion of IEP within twenty four hours compared to the four to five days required pre-

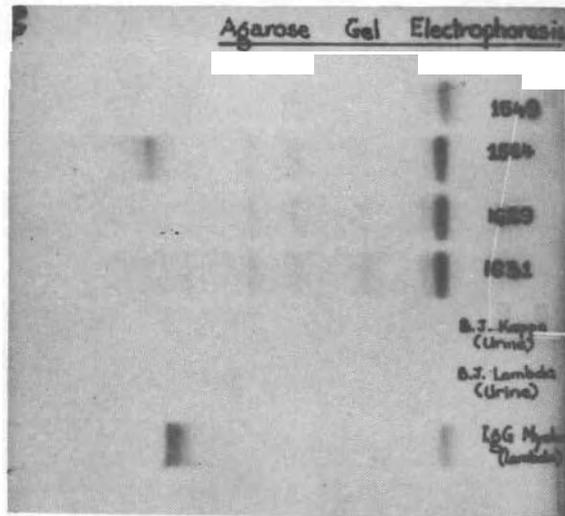
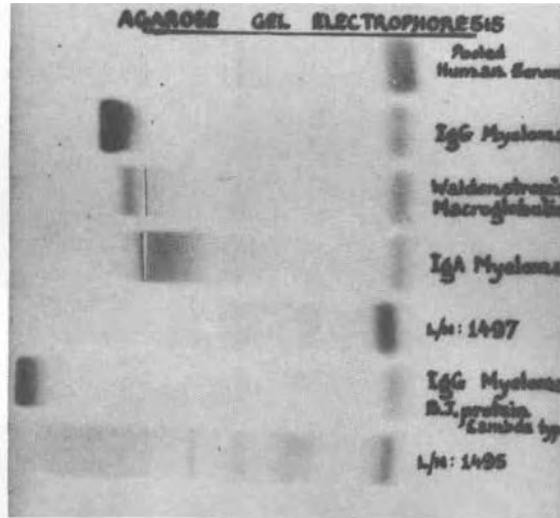


Figure 1: Agarose gel electrophoresis showing monoclonal bands (albumin band is to the right) from various cases of plasma cell dyscrasias and Bence Jones proteinuria. Three closely migrating bands are distinguishable in no. 1564.

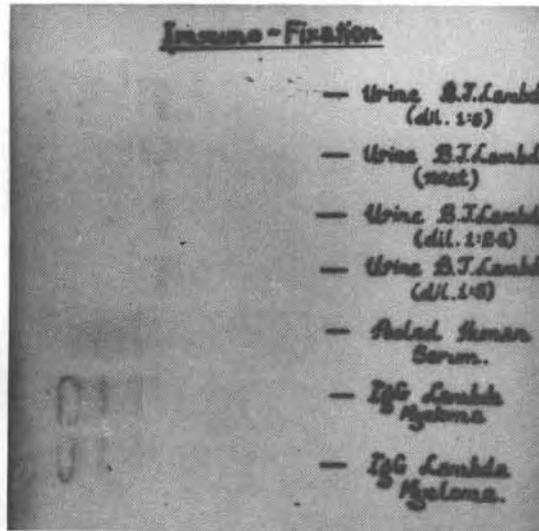


Figure 2: Immunofixationshowingprozone phenomena (BJ lambda neat, IgG lambda myeloma).

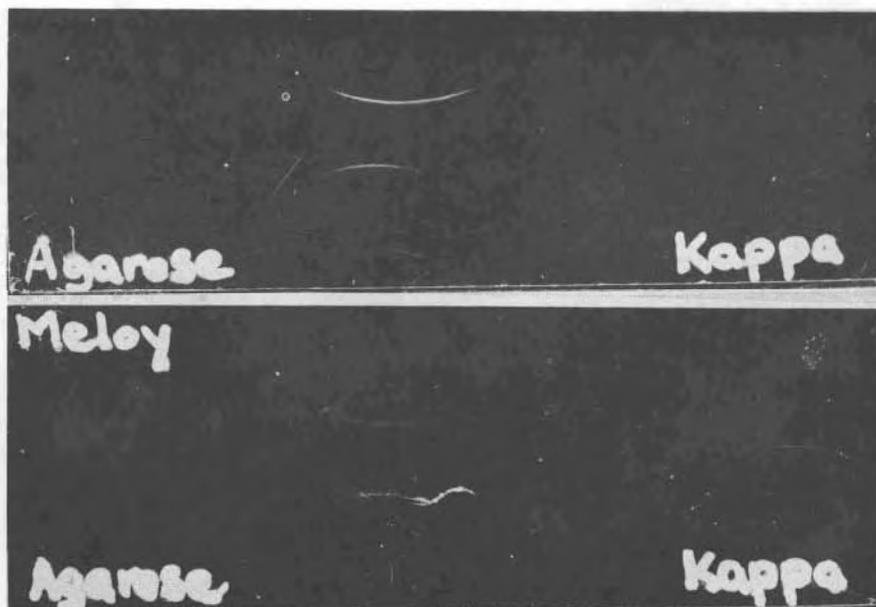


Figure 3: Comparison of Behring and Meloy kappa antisera (IgG kappa myeloma) in one patient (1710). Standard precipitin arc above well, patient below.

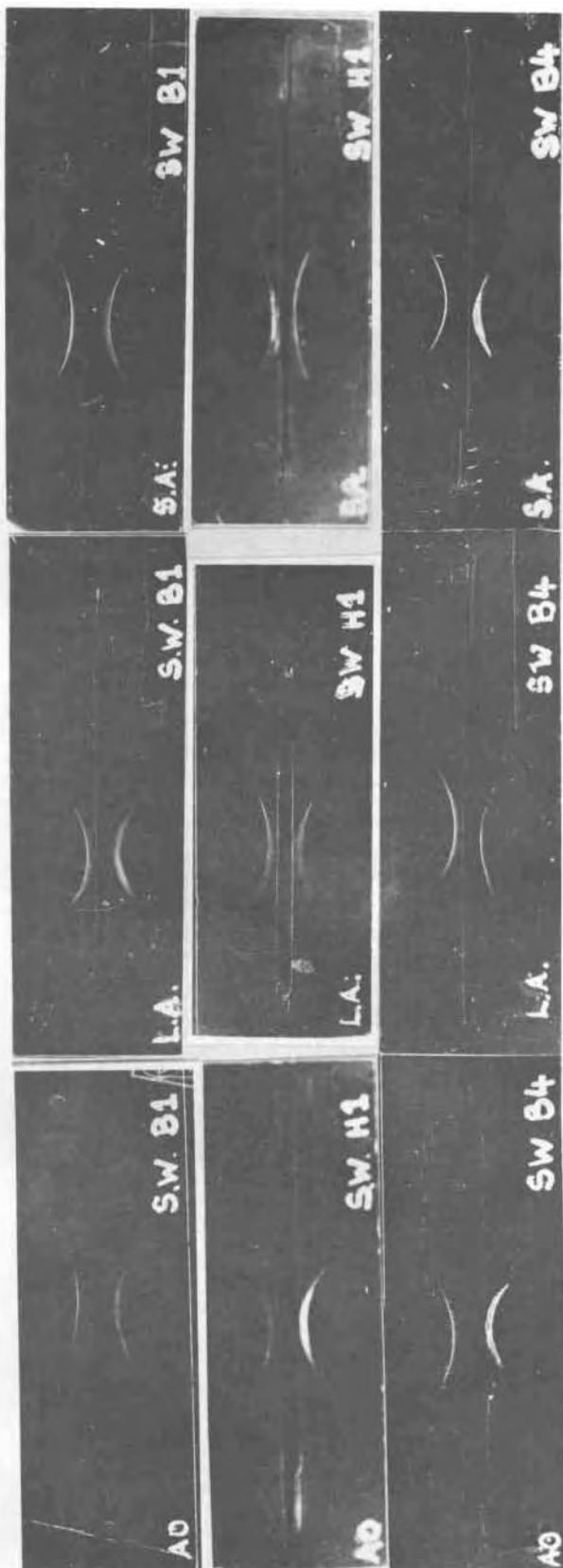


Figure 4: IgG kappa myeloma (SW): comparison of different agaroses and antisera (see text for coding).

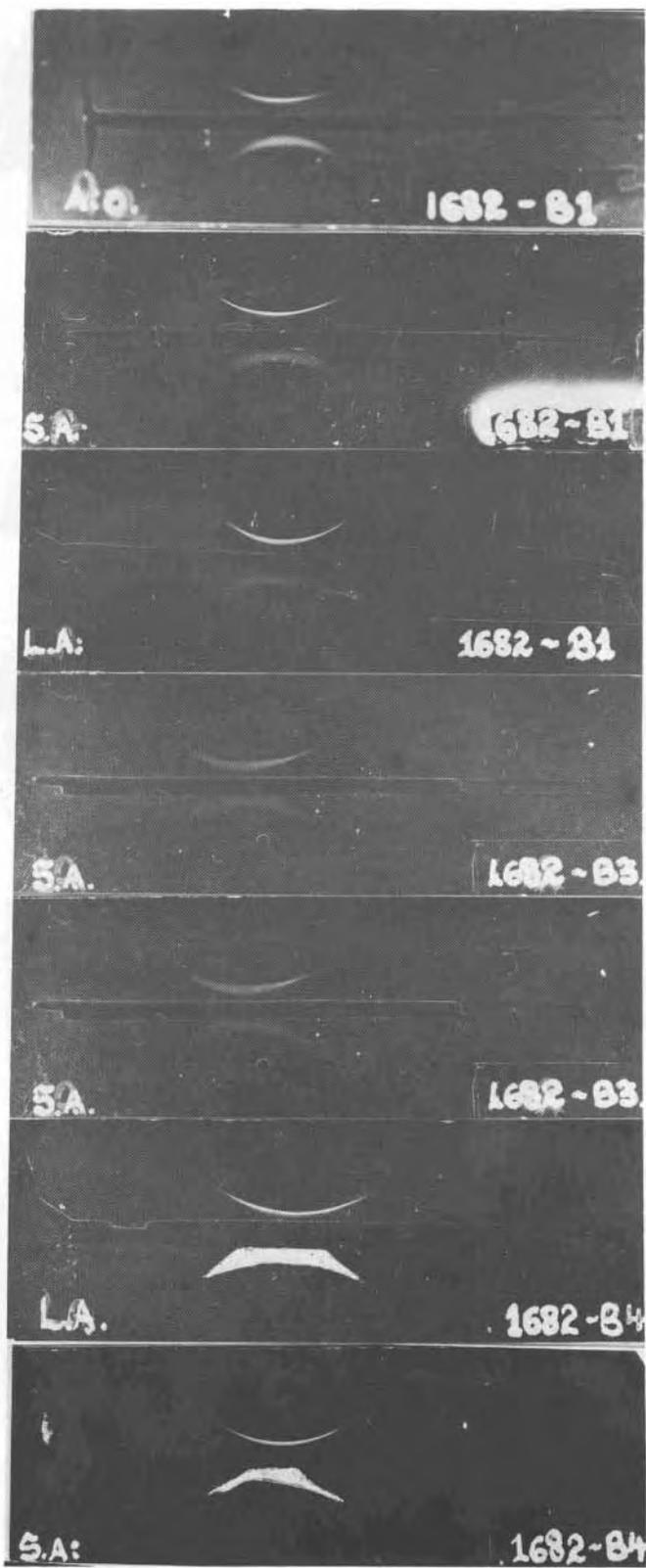


Figure 5: *IgG kappa myeloma (1682): comparison of different agaroses and antisera (see text for coding).*

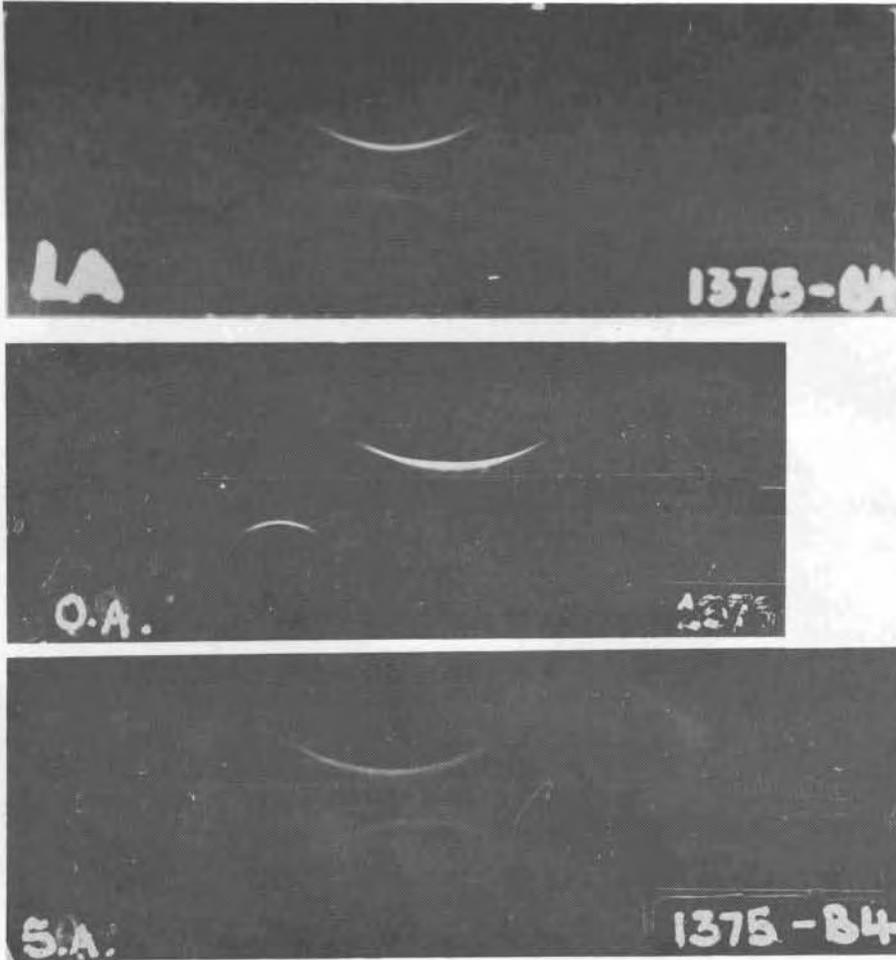


Figure 6: Identification of abnormal kappa (1375) in Oxoid purified agar using Behring anti-bound kappa antiserum.



Figure 7: *Mu chain monoclonal gammopathy* best identified with *Helena antisera* in *Berhing agarose L.*

viously; however, some light background staining occurred but it did not interfere with the reading of the precipitin arcs.

The Bradshaw ring test for urinary globulins is the best of the usually unreliable chemical methods for Bence Jones proteins detection but it fails to detect about 1:20 demonstrable by concentration and electrophoresis.⁹ Immunofixation was very useful in identifying free light chains in urine and serum (Fig. 2 urine BJ lambda) and was more sensitive than IEP. However, the development of Bence Jones precipitin lines in urine or free light chains in serum on IEP showed that it was necessary to read every three hours to prevent missing of precipitin lines due to prozoning (Fig. 8). Concentration of urine was also necessary in some cases (Fig. 9). This may explain the varying incidence of free light chains in serum or urine reported in the literature.

At present we are further comparing the methods of IMF and AGE to IEP to determine which will be performed routinely for screening sera for MIg. Most of our patients in the University Hospital present with the classical Kahler triad and/or are in an advanced stage of the disease. Hence it may be argued that the extra-sensitivity of IMF may not be required for diagnosis. The results from the two series of 69 and 15 patients would seem to speak against this. Furthermore, more discrete bands have been seen with IMF, particularly free light chains, the presence of which would seem to correlate with poorer prognosis. Prozoning was often seen with IMF but was not difficult to identify (Fig. 2) as has already been reported¹⁰ dilution of patient's sera up to 1:50 prevents this. The different iso-electric points of free lambda/kappa chains to the complete parent MIg makes their identification very simple with antisera reacting both with bound and free light chains. In many cases, however, confirmation using antisera monospecific for free kappa or lambda light chains Fab, Fc or Fd fragments and/or such proteins as albumin may be required as the band may be due to the presence of MIg-complexes with other proteins and/or their fragments.

The use of agarose gel bridges as described under IMF shortened the electrophoretic run to

about 50 minutes compared to the usual 60 - 80 minutes due to an increased amount of volts per cm. when compared to the use of Imm thick filter paper as wicks.

The choice of agarose for AGE was critical. Sigma agarose showed marked endo-osmosis and in one case we missed a very cathodal migrating IgG lambda MIg which, however, was identifiable using Behringwerke agarose T but not L.

Polyclonal immunoglobulins can be satisfactorily measured by immunochemical means such as radial immunodiffusion (RID), however, RID is subject to well-documented discrepancy when the test samples assayed contain MIg of the class being determined.^{2, 13} In all our cases of PCD with MIg except for light chain disease, the concentrations of the MIg was always above the upper limit of normal for that class 'X' when determined by RID, however, the concentration was reported as being greater than 'X' and not given as a mg/dl concentration for this reason. The simplest and most practical method when monitoring the patient's progress is to measure the height of the MIg spikes when present after running CAE or AGE through a densitometer.

CONCLUSIONS

Most MIg and their fragments can be determined and typed by IEP, however, CAE cannot be recommended for the screening of sera for MIg and should be replaced by AGE. It is also recommended that when other evidence is indicative of PCD but evidence of MIg is lacking in serum or urine that the sample be subjected to IEP using a variety of gel media and antisera from different commercial sources as well as IMF. The findings question theoretically whether the so-called non-secreting PCD are in fact labelled such because of the limitations of methods used to determine the presence of MIg and whether the resolution power of IEP is considered poor because of the lack of flexibility in its use. Further investigations are planned to try and answer these questions.

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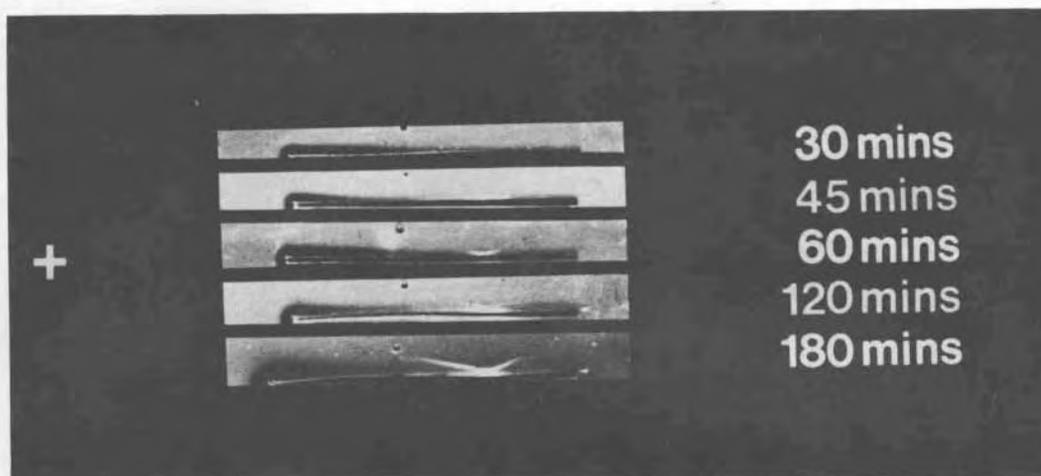


Figure 8: Effect of time on development of immunoprecipitate for kappa Bence Jones proteinuria.

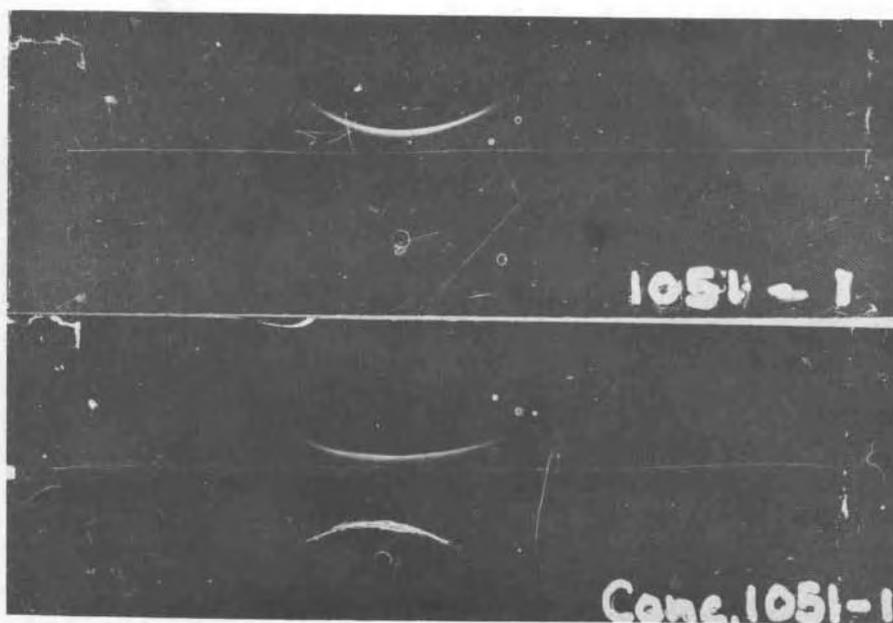


Figure 9: Effect of urine concentration to show kappa Bence Jones proteinuria.

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